Immunochemical and Disc Electrophoresis Study of Soybean Trypsin Inhibitor SBTIA-2

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ABSTRACT

The immunochemical properties of the soybean trypsin inhibitor SBTIA-2 were studied by double gel diffusion, immunoelectrophoresis, single immunodiffusion, and complement fixation with the use of an antisoybean water-extract serum 106. As little as 0.03 microgram of the inhibitor can be detected by the micro complement fixation method with 400-fold dilution of antiserum. The inhibitor was found to be immunochemically identical with a corresponding protein in the total soybean water extract, and thus it is not an artifact of isolation. Stoichiometric addition of trypsin greatly reduces the antigenic sites of the inhibitor as examined by complement fixation. The single immunodiffusion technique can be used for quantitative estimation of inhibitor in the range of 0.02 to 1.00 mg. of the compound. By disc electrophoresis, impurities present in the commercial preparation of the inhibitor exhibit distinctly different patterns in presence and absence of persulfate catalyst, and in gels of various polyacrylamide concentrations. Microdensitometer tracings of the stained bands showed that the inhibitor contained the following amounts of apparent impurities in gels polymerized as indicated: 4% gels, ammonium persulfate, none; 7%, riboflavin and light, 5%; 7%, ammonium persulfate, 43%; and 11%, riboflavin and light, 22%. The possibility of artifact formation caused by the persulfate catalyst is discussed.

Methods for detecting soybean trypsin inhibitors are usually based on the ability of these proteins to inhibit trypsin or chymotrypsin (1). The electrophoretic behavior of various soybean trypsin inhibitors on polyacrylamide gel has been reported by Eldridge et al. (2) and by Frattali and Steiner (3). However, immunochemical methods which offer specificity and high sensitivity have not been utilized in the study of the inhibitors. The objectives of this investigation were to demonstrate the antigenicity of the soybean trypsin inhibitor SBTIA-2, to develop immunochemical methods for its qualitative and quantitative detection, and to examine the trypsin-trypsin inhibitor complex immunologically.

The soybean trypsin inhibitor used in this study was a commercial preparation obtained according to the procedure of Rackis et al. (4) and corresponds to their chromatographic component VI. This component is assumed to be identical with the classic inhibitor described by Kunitz (5). Attempts to determine the degree of purity of the commercial preparation by disc electrophoresis led to highly heterogeneous results. It was found that the amount of impurities that can be demonstrated in the preparation varies with the concentration of the polyacrylamide in the gel and the mode of polymerization of the gel.

MATERIALS AND METHODS

Materials

Soybean trypsin inhibitor preparations (lots T-1005 and T-2178) were purchased from Mann. Trypsin 2X crystallized (lot TF185F) was obtained from Worthington.

Total water-extractable soybean proteins were prepared as follows: soybeans, Harosoy 63 variety grown in 1964 and stored at 25°C., were cracked, dehulled, and flaked. The soybean flakes were defatted with pentane and desolventized at room

[1] N. Catsimpoolas and E. W. Meyer, Separation of soybean whey proteins by isoelectric focusing (MS in preparation).

temperature. The defatted flakes were extracted with water (flake:water ratio, 1:5) at 25° C. and centrifuged at 10,000 × g to clarify the supernatant liquor.

Preparation of Antisera

The total soybean water-extract, mixed and homogenized with an equal volume of Freund's complete adjuvant (Difco), was used for intraperitoneal immunization of New Zealand White rabbits. The immunizing dosage was 1 ml. the first week, 2 ml. the second week, and 5 ml. the third week. After a 30-day rest period, the rabbits were given a 5-ml. booster injection. The animals were then subjected to test bleeding from the marginal ear vein. If the concentration of precipitating antibody was adequate, the rabbits were exsanguinated and the sera were stored at 4° C. after filtration sterilization and addition of 1:10,000 merthiolate. Antiserum 106 was prepared by this method.

Immunochemical Methods

Double gel diffusion in agar was carried out in plates according to the method of Ouchterlony (6). The gel medium consisted of a 0.85% Ionagar No. 2 (Oxoid) solution in pH 6.8 phosphate buffer prepared as follows: 0.77 g. sodium phosphate dibasic, 0.38 g. potassium phosphate monobasic, and 10.02 g. sodium chloride were dissolved in water and made up to 1-liter volume after addition of 1 ml. of 1% merthiolate solution. The reactants were allowed to diffuse at room temperature.

Immunoelectrophoresis in agar gel was carried out by the general procedure described by Grabar and Williams (7) as modified by Scheidegger (8). The gel medium consists of 1% Ionagar No. 2 (Oxoid) in pH 8.8 tris-barbital-sodium barbital, 0.05 ionic strength buffer (Gelman). Electrophoresis was carried out for 2.5 hr. with a current of 5 ma. per microscope slide. Staining of the precipitin arcs was done with Ponceau S as described by Uriel (9).

A standard curve for quantitative immunochemical determination of soybean trypsin inhibitor was developed by use of a single-diffusion technique. Glass tubes, 2 mm. i.d. and approximately 100 mm. long, were coated with 0.1% Ionagar (Oxoid) and dried at 70° C. For the single-diffusion procedure, these tubes were half-filled with 1% Ionagar in pH 7.0 buffered saline (8.713 g. sodium phosphate dibasic heptahydrate, 2.313 g. sodium phosphate monobasic monohydrate, and 8.775 g. sodium chloride, made to 1-liter with water) containing merthiolate (1:10,000) and antisoybean water-extract serum 106 at a final dilution of 1:2. Soybean trypsin inhibitor dispersed in the above-described gel diffusion buffer (0.2 ml. containing from 5 micrograms to 1 mg. of inhibitor) was layered above the gel and the tubes were capped with Vaspar (50:50 white petroleum: paraffin). Migration of the antigen-antibody precipitate was measured with a measuring magnifier after incubation of the tubes at 20° C. for 24 hr.

Reagents and procedures for the micro complement (C') fixation technique are described by Levine and Van Vunakis (10).

Disc Electrophoresis

Polyacrylamide gel electrophoresis in tris-glycine buffer was carried out essentially as described by Davis (11). The method is also described in detail in the instruction manuals of Canal Industrial Corporation. Alternatively, riboflavin and light were used to polymerize the gels, as described by Brewer (12) when persulfate was omitted.

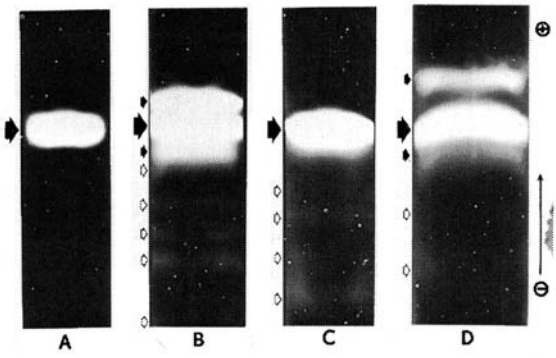


Fig. 1. Disc electrophoresis migration patterns of commercial soybean inhibitor SBTIA-2: A, 4% polyacrylamide gels polymerized with ammonium persulfate catalyst; B, 7% gels polymerized with ammonium persulfate; C, 7% gels polymerized with riboflavin and light; D, 11% gels polymerized with riboflavin and light. Solid arrows indicate major bands and open arrows, minor bands.

Densitometric tracings and integration of areas under the trace were performed with a Canalco Model F microdensitometer.

RESULTS AND DISCUSSION

Disc Electrophoresis

Figure 1, A, shows the disc electrophoresis pattern of commercial soybean trypsin inhibitor in 4% gel polymerized with persulfate. Only one major band is seen. At this concentration of gel, proteins are separated mainly by charge. The "sieving" effect of the gel pores on proteins with molecular weights of 14,000 to 24,000, which is the approximate range of molecular weights that the various inhibitors exhibit, can be assumed to be insignificant. Thus, protein impurities of charge similar to that of the SBTIA-2 inhibitor but of different molecular size cannot be detected in this type of gel. When the concentration of the acrylamide in the gel is increased to 7% and persulfate is used as the catalyst, the pattern shown in Fig. 1, B, was obtained. Besides the band of the SBTIA-2 inhibitor (indicated by a large solid arrow), two other major and at least five minor bands are present. Integration of the area occupied by each band under the densitometer trace indicated the following approximate composition: fast-moving band, 16%; SBTIA-2 inhibitor, 57%; slower-moving bands, 28% of the total area.

When the trypsin inhibitor was electrophoresed on 7% gel polymerized by riboflavin and light (12), the results shown in Fig. 1, C, were obtained. The inhibitor exhibited one major and a few minor slower-moving bands only. The major band represented 95% of the total densitometer tracing area. Figure 1, D, shows the disc-electrophoresis pattern of the inhibitor in 11% gel polymerized with riboflavin and light. In this case, the major band represented 78% of the total area. Although the gels prepared under different experimental conditions are different in length and diameter after electrical destaining with 7% acetic acid, the relative mobility of the major band in the separating gel remains the same when the tracking dye is allowed to migrate a fixed distance (10 mm. from the anodic end of the column) during electrophoresis.

Artifact formation by the use of ammonium persulfate as a catalyst for gel polymerization has been discussed by other investigators (12,13,14). Ornstein (15) suggested that the persulfate may never come in contact with the protein, because it has far greater mobility in the gel than any protein. However, it eliminates all reducing agents, thus leaving the gel in an oxygen-rich state. The formation of artifacts in the presence of persulfate can very conveniently explain the high degree of heterogeneity obtained by disc electrophoresis of the trypsin inhibitor in 7% gels. Although artifact formation is possible, the authors also noticed that gels polymerized with riboflavin and light do not exhibit the same firmness as gels polymerized with persulfate. Since the firmness of the gel is associated with the degree of cross-linking, loss of resolution may occur, leading to incomplete separation of bands (Fig. 1, C). This can be remedied by increasing the concentration of the gel to 11% or higher (Fig. 1, D). The 11% gel polymerized with riboflavin and light has the advantage of improved resolution and avoidance of persulfate catalyst. Results obtained with this type of gel are probably more representative of artifact-free heterogeneity patterns. When this gel was used, the inhibitor was found to contain 22% of impurities. This value is in reasonable agreement with results obtained by Frattali and Steiner (3), which indicated that a commercial preparation of crystalline trypsin inhibitor contained 23% impurities. The major band (F_2) obtained by polyacrylamide-gel electrophoresis was 77% of the total area and was found identical to the SBTIA-2 inhibitor. It is possible that the faster- and slower-moving bands of the major impurities as revealed in the 11% gel (Fig. 1, D) correspond, respectively, to the inhibitors F_3 and F_1 obtained by Frattali and Steiner (3).

Antigenicity of Soybean Trypsin Inhibitor

In the course of studies on the characterization of soybean proteins by immunochemical methods (16,17,18,19), attention was drawn to an antisoybean water-extract serum 106 which contained a relatively high concentration of trypsin inhibitor antibodies. This relative specificity is due to the individual variations in antibody response of experimental animals which is commonly observed in immunization procedures. This antiserum reacts with several soybean globulin components, but it was found specific for the trypsin inhibitor SBTIA-2 when reacted with the total soybean whey protein fraction. No other bands were observed. This was ascertained by the reaction of antiserum 106 with soybean whey protein components separated by isoelectric focusing[1]. Although several isolated components exhibited trypsin inhibitory activity, probably corresponding to other inhibitors present in soybean extracts, only the inhibitor isolated at the pI (isoelectric point) range 4.45 to 4.53 (peak at pI 4.47) and exhibiting a disc-electrophoresis pattern identical with the major band of the commercial SBTIA-2 inhibitor gave an immunoprecipitin band with antiserum 106. Consequently, this antiserum was used for all the immunochemical experiments reported here.

Double Gel Immunodiffusion

Examination of the inhibitor by double gel immunodiffusion (Fig. 2) indicated that a precipitin reaction occurred between this protein and its antibody. Because of the high diffusion rate of the inhibitor in relation to the immunoglobulin, the immunoprecipitin band (see arrow) is formed in close proximity to the antiserum

well. This band is usually visible within 5 to 16 hr. of diffusion time, and on prolonged diffusion may disappear, especially when the antigen and antibody are not present at concentrations close to the optimum ratio. The reasons for reversal of the antigen-antibody aggregation have been discussed in detail by Crowle (20). A similar band is formed when the total water-extractable protein fraction is diffused against the antiserum. This band coalesces (fuses) with the band of the inhibitor, indicating immunochemically identical molecules. Thus, the antiserum can be used to detect the inhibitor in soybean extracts. The coalescence of proximal band tips also shows that the antigenic sites of the inhibitor are not altered during the isolation procedure. The slower-moving antigenic components of the total water-extractable proteins usually appear within 24 to 48 hr. of diffusion and are due to globulin components which are isoelectrically precipitated at pH 4.5. The inhibitor remains in the whey fraction.

Since several soybean trypsin inhibitors have been reported present in soybeans (1,3,4,21,22,23), it would be of interest to examine the antigenicity of all the isolated inhibitors. When such specific antisera become available, the detection and, perhaps, quantitation of the various inhibitors will be much simplified. Such methods can also be used to reveal the function of the inhibitors in the metabolic processes of the germinating soybean.

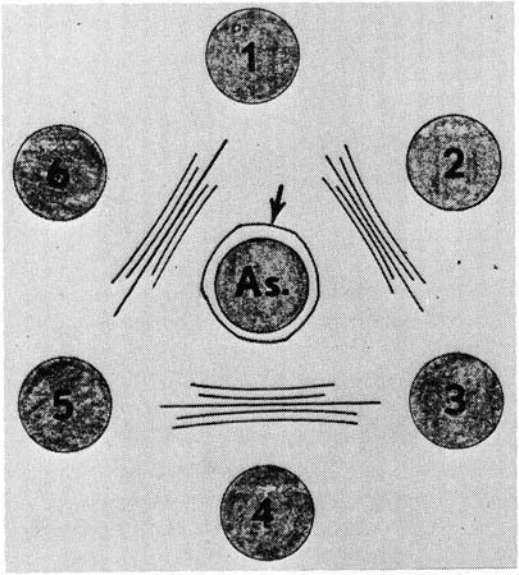


Fig. 2. Schematic diagram of double gel immunodiffusion patterns developed with antiserum 106. Center well: antiserum 106; wells 1, 3, and 5: soybean trypsin inhibitor SBTIA-2; wells 2, 4, and 6: total soybean water-extract. Arrow indicates the soybean trypsin inhibitor immunoprecipitin band.

Immunoelectrophoresis

Figure 3 shows the results obtained by subjecting the inhibitor and the total water-soybean extract to immunoelectrophoresis in agar gel. The inhibitor exhibited one immunoprecipitin arc proximal to the antiserum trench. A corresponding arc was demonstrated in the total water-soybean extract. These arcs

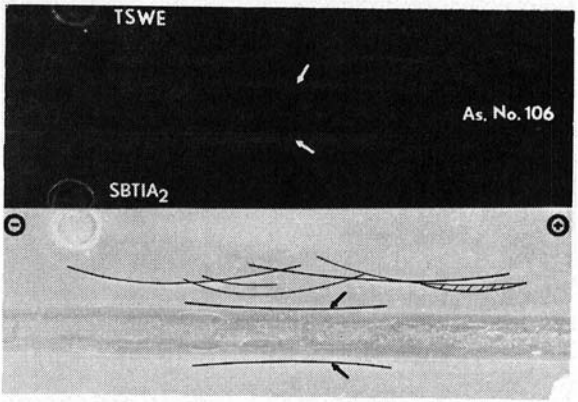
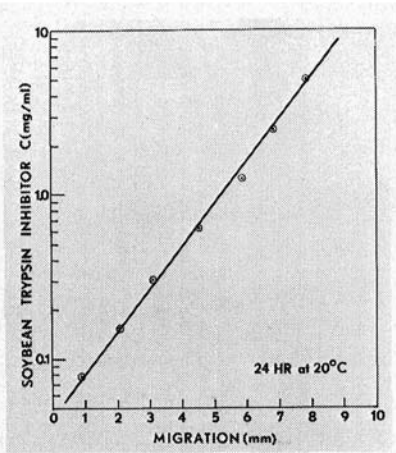


Fig. 3. Actual photograph and schematic diagram of immunoelectrophoresis patterns obtained from total soybean-water extract (TSWE) and soybean trypsin inhibitor SBTIA-2 developed with antiserum 106. Arrows indicate the position of the soybean trypsin inhibitor immunoprecipitin arcs.

fused by the interrupted-trough technique (24), indicating immunochemical identity of the inhibitor with the corresponding protein present in the total soybean extract. The immunoprecipitin arcs corresponding to the inhibitor were visible within 4 to 5 hr. of diffusion, whereas the remaining bands of the total extract were usually formed after 16 hr. of diffusion. The immunoelectrophoresis technique may be useful in immunochemical characterization of other soybean trypsin inhibitors, since they exhibit different electrophoretic mobilities (23,25).

Single Immunodiffusion

Immunodiffusion methods which can be used to detect an antigen can also be employed for quantitation. Single immunodiffusion techniques have been used to quantitate a number of proteins (20). In a previous study (17) we described a method based on single diffusion for the quantitative immunoassay of the 11S component (26) of soybean globulins. Figure 4 shows the standard curve obtained from single diffusion experiments of soybean trypsin inhibitor in agar gel containing antiserum 106. The log of soybean trypsin inhibitor concentrations (in mg. per ml., uncorrected for the presence of impurities) approximated a straight line when plotted against the distances of migration of the precipitin bands (in mm. at 20°C.,for a 24-hr. diffusion period. Concentrations of the inhibitor in the range of 0.02 to 1 mg. (0.2 volume is used for the assay) can thus be estimated. Lower concentrations of the inhibitor can be determined by increasing the diffusion time; however, the position of the precipitin bands cannot be visualized with accuracy. It must be realized that this method can be applied only with an immunochemically homogeneous inhibitor when an antisoybean extract antiserum is used. Determination of the inhibitor in a soybean extract will require an antiserum specific for the inhibitor. In the latter case, the sensitivity of the method may be improved by the presence of high amounts of trypsin inhibitor antibodies. Such specific antisera could be used for quantitation of the various inhibitors. However, the scope of the present work was to develop the principle of a single diffusion technique which would be used with a number of inhibitors and antisera. Of course, a new standard curve will have to be determined for each system.



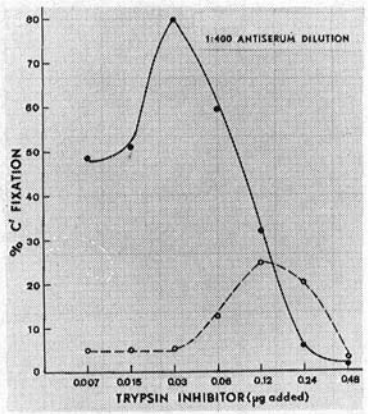


Fig. 4(left). Single diffusion of soybean trypsin inhibitor against antiserum 106. Migration of precipitin bands obtained from different concentrations of the inhibitor at 24 hr. of diffusion at 20° C.

Fig. 5(right). Micro complement (C') fixation curves obtained with a 400-fold antiserum dilution from different concentrations of the soybean trypsin inhibitor alone (solid line) and in the presence of a stoichiometric amount of trypsin (dotted line).

Complement Fixation

The soybean trypsin inhibitor can be detected with great sensitivity by the technique of micro complement fixation (10). The principle of this method is based on the complement system of guinea-pig serum which has the capacity to combine irreversibly with antigen-antibody complexes. The combination can be estimated by the residual hemolytic activity of the complement when mixed with sensitized sheep erythrocytes. It can be seen from Fig. 5 that as little as 0.03 microgram of the inhibitor is required for maximum complement fixation (point of optimum antigen-antibody reaction) with a 400-fold dilution of the antiserum. This method is extremely useful when the amount of purified protein available for analysis is very minute or the supply of antiserum is limited. Antigen-antibody reactions can be demonstrated by the typical shape of the curve in Fig. 5. Maximum reaction is obtained at the optimum ratio of antigen to antibody. Excess of antigen or antibody tends to resolubilize the antigen-antibody complex, with consequent diminished complement fixation.

Levine and Van Vunakis (10) have discussed the sensitivity of the complement fixation method in detecting local conformational changes of antigenic determinants in a protein molecule or changes due to the interaction of proteins. When the trypsin inhibitor was examined, in the presence of a stoichiometric amount of trypsin (27), by the complement fixation method, both a lateral and a vertical shift of the fixation were observed (Fig. 5). This indicates both an alteration of the antigenic determinants of the inhibitor in the presence of trypsin (vertical shift), and a more general conformational change of the modified molecule (lateral shift) (10). The unavailability of the antigenic sites of the inhibitor may be partly the result of steric hindrance. This can be visualized as a blocking of the antigenic sites of the inhibitor by the trypsin, especially in the undissociated complex. However, the cleavage of the Arg-Ileu peptide bond of the inhibitor by trypsin (28) may be

accompanied by a conformational change which destroys antigenic sites dependent on the conformation stability of the native molecule. The possibility that trypsin digests the gamma-G immunoglobulins can be excluded because of the presence of the inhibitor. In addition, Arnon and Schechter (29) have shown in the trypsin-anti-trypsin system that the rate of antigen-antibody complex formation is much higher than the potential enzymatic degradation of the gamma-G globulins.

The availability of specific antibodies for the various isolated inhibitors would offer simple and rapid methods for their detection and possible quantitative determination, and a means for study of their interrelationships. Immunochemical methods may show whether the inhibitors are present in soybean extracts obtained under minimum denaturation conditions or are artifacts of isolation. We have shown that the classic inhibitor is present in water-extracts of defatted soybean flakes in a state immunochemically identical with that of the isolated inhibitor, SBTIA-2.

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